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G Protein-Coupled Receptor Kinase 5 Phosphorylation of Hip Regulates Internalization of the Chemokine Receptor CXCR4

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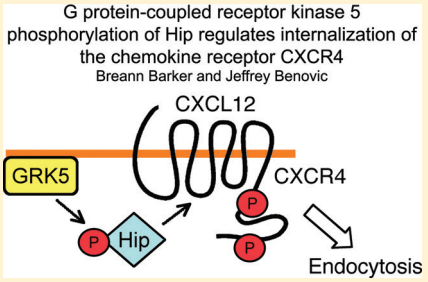
ABSTRACT: Regulation of the magnitude, duration, and localization of G protein-coupled receptor (GPCR) signaling responses is controlled by desensitization, internalization, and downregulation of the activated receptor. Desensitization is initiated by the phosphorylation of the activated receptor by GPCR kinases (GRKs) and the binding of the adaptor protein arrestin. In addition to phosphorylating activated GPCRs, GRKs have been shown to phosphorylate a variety of additional substrates. An *in vitro* screen for novel GRK substrates revealed Hsp70 interacting protein (Hip) as a substrate. GRK5, but not GRK2, bound to and stoichiometrically phosphorylated Hip *in vitro*. The primary binding domain of GRK5 was mapped to residues 303−319 on Hip, while the major site of phosphorylation was identified to be Ser-346. GRK5 also bound to and phosphorylated Hip on Ser-346 in cells. While Hip was previously implicated in chemokine receptor trafficking, we found that the phosphorylation of Ser-346 was required for proper agonist-induced internalization of the chemokine receptor CXCR4. Taken together, Hip has been identified as a novel substrate of GRK5 *in vitro* and in cells, and phosphorylation of Hip by GRK5 plays a role in modulating CXCR4 internalization.



G protein-coupled receptors (GPCRs) elicit a variety of cellular responses via activation of downstream signaling pathways. Tight control of the magnitude, duration, and location of these signaling responses is critical for homeostasis. The mechanisms regulating receptor signaling include desensitization, internalization, and downregulation. Desensitization, the process by which cells become unresponsive to repeated stimulation, is initiated by phosphorylation of the receptor by GPCR kinases (GRKs) and second messenger kinases, such as protein kinase A (PKA) and PKC. Phosphorylation of the receptor by GRKs recruits arrestins, further leading to receptor desensitization. Internalization, whereby receptors are removed from the cell surface, is largely mediated by clathrin-adaptor proteins, such as arrestins. Downregulation, a decrease in the total number of receptors, may involve targeting GPCRs to the lysosome by ubiquitin-dependent interactions with components of the lysosomal sorting machinery.[1−3]

GRKs are serine/threonine protein kinases that preferentially phosphorylate agonist-occupied GPCRs. The GRK family is composed of seven members, GRK1−7, and subdivided into three subclasses based on homology: GRK1 and -7, GRK2 and -3, and GRK4, -5, and -6.[4,5] Although all of the GRKs have distinct regulatory mechanisms, all GRKs consist of an N-terminal domain that contains a regulator of G-protein signaling homology domain, a central kinase domain that is related to AGC kinases, such as PKA and PKC, and a C-terminal domain that is the most variable.[4,5] The C-termini of GRK2 and -3 contain a pleckstrin homology domain that binds phospholipids and G$\beta\gamma$ subunits and mediates plasma membrane recruitment upon receptor activation,[6−8] the C-termini of GRK4 and -6 are palmitoylated, which contributes to their plasma membrane localization,[9−11] and the C-terminus of GRK5 directly binds lipids.[12,13]

Work examining GRK2 knockout mice and various cellular studies led to the search for nonreceptor substrates of GRKs. Initial studies identified tubulin as the first nonreceptor substrate of GRKs.[14,15] Subsequent studies identified Smoothened,[16,17] PDGF receptor,[18] EGF receptor,[18] synucleins,[19] phosducin,[20] PDEγ,[21] R-Smads,[22] ezrin,[23] NFκB1 p105,[24] p38,[25] downstream regulatory element antagonist modulator (DREAM),[26] histone deacetylase 5 (HDAC5),[27] arrestin2,[28] and p53.[29] The majority of these substrates were found to be substrates of GRK2 and were not found using a systematic approach.

In order to identify novel nonreceptor substrates of GRK5, a bovine brain lysate was screened for GRK5 substrates using an *in vitro* kinase assay. One of the novel substrates was identified as Hsp70 interacting protein (Hip), which functions as a cochaperone to other heat shock proteins, such as Hsp70 and Hsp90, to mediate protein folding, assembly of multiprotein complexes, and transport of proteins within the cell. Here, we show that Hip is a novel nonreceptor substrate and binding partner of GRK5 *in vitro* and in cells. GRK5 phosphorylates Hip primarily at Ser-346, and phosphorylation of this residue modulates the ability of Hip to mediate internalization of the chemokine receptor, CXCR4.

MATERIALS AND METHODS

Cell Culture and Transfections. HEK293 and HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum.

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HEK293 cells ~70% confluent in 10 cm plates were transiently transfected with 600 pmol of siRNA using Lipofectamine2000 (Invitrogen) for 72 h as per the manufacturer's instructions. HEK293 and HeLa cells were transiently transfected with 6 μg of plasmid DNA using Fugene6 (Roche) for 48 h. Cells stably expressing an shRNA against Hip were selected and maintained in DMEM containing 10% fetal bovine serum and 100 μg/mL of hygromycin.

Hip wild-type and domain deletion cDNAs were generously provided by Dr. Marc B. Cox from the University of Texas at El Paso in a pSPUTK vector. Hip was then subcloned into pTrcHisB vector using *Bgl*II and *Kpn*I, into pGEX4T using *Sal*I and *Eco*RI, and into pcDNA3 using *Eco*RI and *Not*I. Point mutations were introduced by site-directed mutagenesis using QuikChange II Site-Directed Mutagenesis Kit (Stratagene) and confirmed using dideoxy sequencing.

siRNA oligos targeting GRK2, -3, -5, and -6 were synthesized by Dharmacon and were previously described.[30] An shRNA vector targeting Hip was a generous gift from Dr. Marc B. Cox from the University of Texas at El Paso. The shRNA targeted the sequence 5′-ACCAGTGTACCTCTGACCT-3′ which spans base pairs 1277−1299 in the 3′ untranslated region of the Hip gene and was inserted into pSilencer hygro vector (Ambion).

Purification of His-Tagged Hip Constructs. Hip expression was induced in BL21(DE3) cells at 30 °C for 4 h using 0.1 mM IPTG. Cells were collected by centrifugation, frozen at −80 °C, and thawed in buffer containing 100 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, and protease inhibitor tablets without EDTA (Roche). The cell lysate was then homogenized, sonicated, and centrifuged at 32000*g*. The supernatant was applied to a Ni-NTA Sepharose column. The column was washed, and the bound proteins were eluted with a 20−500 mM gradient of imidazole. Peak fractions were pooled, concentrated, and applied to a gel filtration column using a buffer containing 20 mM Tris-HCl, pH 8.0, 5 mM EDTA, 50 mM NaCl, and 1 mM dithiothreitol. Peak fractions were again pooled, concentrated, and frozen at −80 °C. The purified protein was analyzed by SDS-PAGE and Coomassie staining, and the concentration was determined using a Bradford assay.

***In Vitro* Kinase Assay.** GRK2 and GRK5 were purified as previously described.[31,32] Hip (100 nM) was incubated with 100 nM GRK5 or GRK2 in 10 mM Tris-HCl, pH 7.5, 2 mM EDTA, 7.5 mM $MgCl_2$, 100 μM ATP, and 1−2 μCi of [γ-^{32}P] ATP for various times at 30 °C while shaking. For assays including crude phosphatidylcholine (PC), PC was resuspended in buffer containing 20 mM Tris-HCl, pH 7.4, 1 mM EDTA, and 50 mM NaCl and sonicated for 10 s at room temperature. PC (0.1 mg/mL) was added to the reaction as indicated. Kinase assays were terminated by the addition of SDS sample buffer. Samples were separated by SDS-PAGE, and the gels were dried onto filter paper and subjected to autoradiography. The amount of [^{32}P] phosphate incorporated was determined by scintillation counting of the excised protein bands.

Glutathione S-Transferase (GST) Pulldown Assays. GST Hip Wt and mutant proteins were expressed in BL21(DE3) cells. Cells were then lysed in buffer containing 20 mM Hepes, 0.02% Triton X-100, 100 mM NaCl, 10 mM EDTA, and a protease inhibitor tablet (Roche) and centrifuged at 16000*g*. Soluble protein was incubated with Glutathione Sepharose beads followed by extensive washing of the beads. The purity of the proteins was assessed by Coomassie staining, and protein concentrations were determined using a Bradford assay. GST Hip proteins (21 pmol) were incubated with 3 pmol of purified GRK2 or GRK5 in total volume of 500 μL of buffer (20 mM Hepes, pH 7.2, 100 mM potassium acetate, and 0.1% Triton) at room temperature for 1.5 h. Beads were washed extensively, and bound protein was eluted by the addition of SDS sample buffer. Proteins were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted for GRK5 using a GRK4−6 monoclonal antibody (Millipore). The amount of bound GRK5 was quantitated using an Odyssey scanner (Li-Cor Biotechnology).

Co-immunoprecipitations. Cells were washed with ice-cold phosphate buffered saline (PBS) and lysed in a buffer containing 50 mM Tris-HCl, pH 7.4, 5 mM EDTA, 50 mM NaCl, 1% Triton X-100, and a protease inhibitor tablet (Roche). Equivalent amounts of cell lysates were immunoprecipitated with a polyclonal anti-HA antibody (Covance). Immunoprecipitates were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with a monoclonal anti-GRK4−6 antibody (Millipore). Blots were then stripped and reprobed with a monoclonal anti-Hip antibody (Novus Biologics). Total cell lysates were probed with anti-Hip, anti-GRK4−6, and anti-α-tubulin (Sigma) antibodies. Immunoblots were incubated with SuperSignal Enhanced Chemiluminescent (Thermo Scientific) substrate, exposed to film, and developed.

Hip Phosphorylation in HEK293 Cells. Cells were washed with ice-cold PBS 72 h post-transfection and lysed in the same lysis buffer described above containing a PhosSTOP tablet (Roche). The HA-tagged Hip was then immunoprecipitated from equivalent amounts of cell lysates using a monoclonal anti-HA antibody (Covance). For experiments utilizing λ-phosphatase (λ-PPase) (New England Biolabs), immunoprecipitates were incubated with 800 U of λ-PPase for 30 min at 30 °C in a buffer containing Mn^{2+}. For experiments requiring CXCR4 activation, cells were serum starved for 30 min and then stimulated with 50 nM CXCL12 for 15 min prior to lysis. Immunoprecipitates were then separated by SDS-PAGE, transferred to low-fluorescence PVDF membranes, and stained with Pro-Q Diamond Stain (Invitrogen) according to the manufacturer's instructions. Phosphorylated Hip was visualized using a Typhoon Scanner (GE Healthcare) and quantified using ImageQuant 5.2 software (GE Healthcare). Membranes were then probed for total Hip using a polyclonal anti-Hip antibody (Cell Signaling).

CXCR4 Internalization Assay. Control or Hip shRNA HeLa cells were transfected with wild-type FLAG CXCR4 and pcDNA, Hip Wt, Hip-S346A, or Hip-S346D plasmid as indicated and assayed 48 h post-transfection. Serum starved cells were stimulated with 50 nM CXCL12 for 30 min at 37 °C, washed with ice-cold PBS, and fixed with 4% paraformaldehyde on ice. The amount of remaining cell surface CXCR4 was determined using a cell surface ELISA. Cells were incubated with M1 anti-FLAG antibody (Sigma), washed, and incubated with a HRP conjugated horse anti-mouse secondary antibody (Vector Laboratories). After washing, cells were incubated with one-step ABTS (Pierce). An aliquot was removed and the absorbance at 405 nm was determined.

Statistical Analysis. All statistics were performed using a Student's *t*-test.

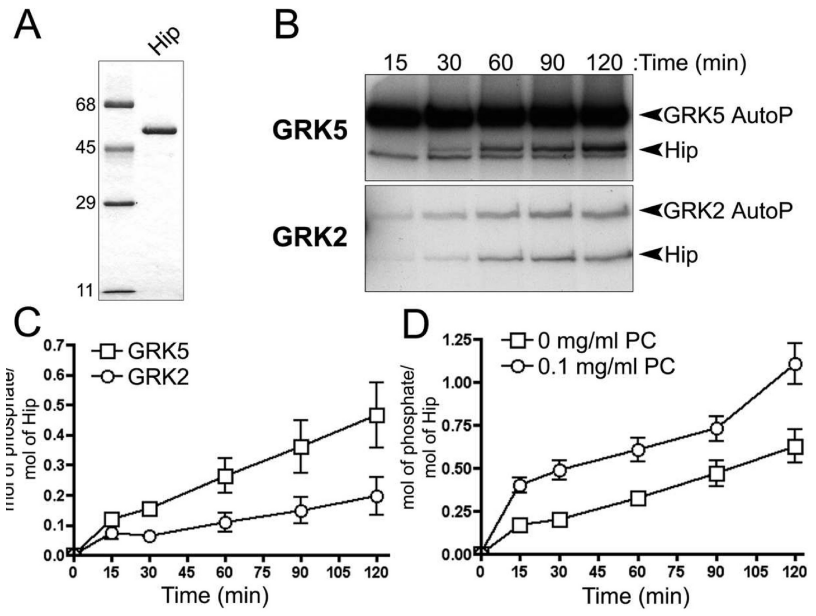

Figure 1. *In vitro* phosphorylation of purified Hip by GRK5. (A) Hexahistidine-tagged Hip was expressed in *E. coli* and purified using Ni^{+} agarose and size exclusion chromatography. 1 μg of purified Hip was separated by SDS-PAGE and Coomassie stained. (B) 100 nM of purified GRK2 or GRK5 was incubated with 100 nM of purified Hip for various times at 30 °C in a buffer containing radiolabeled ATP. Samples were separated by SDS-PAGE and subjected to autoradiography. (C) The radioactively labeled bands from (B) were excised and quantitated by scintillation counting to determine the stoichiometry of phosphorylation. Data represent the mean ± SD from six separate experiments. (D) Mixed micelles were generated by sonication of resuspended PC. The *in vitro* kinase assays were performed as in (B, C) in the presence or absence of 0.1 mg/mL PC vesicles. Data represent the mean ± SD from four separate experiments.

RESULTS

Identification of Hip as a GRK5 Substrate. To identify potential novel GRK substrates, a bovine brain lysate was screened for proteins that are phosphorylated by GRKs. A crude bovine brain lysate was initially resolved using Q sepharose chromatography, and fractions were subjected to *in vitro* phosphorylation in the presence or absence of purified GRK2 or GRK5. This analysis identified an ~50 kDa protein that was phosphorylated in a GRK5-dependent manner. The fraction was further separated using size exclusion chromatography, which resulted in the identification of a single Coomassie stained band that was phosphorylated by GRK5. Mass spectral analysis of this band identified 20 unique peptides of which 13 were mapped to Hsp70 interacting protein (Hip) providing 13.6% sequence coverage (data not shown).

To confirm that Hip was a substrate of GRK5 *in vitro*, hexahistidine-tagged Hip was expressed in *E. coli*, purified using Ni^{+} agarose and gel filtration chromatography (Figure 1A), and subjected to *in vitro* phosphorylation by GRK2 and GRK5 (Figure 1B). GRK5 phosphorylated Hip with a stoichiometry of ~0.5 mol of phosphate per mole of Hip, while GRK2 phosphorylated Hip to a significantly lesser extent (Figure 1C). Since the *in vitro* phosphorylation of Hip by GRK5 was slow, modulators of GRK5 activity, including phosphatidylcholine (PC) and calmodulin, were tested for their ability to enhance phosphorylation of Hip. Addition of PC significantly enhanced the rate of phosphorylation and increased the stoichiometry to 1 mol of phosphate per mole of Hip (Figure 1D). In contrast, the addition of calmodulin inhibited Hip phosphorylation (data not shown).

To determine if Hip binds to GRK5, we used GST pulldowns and co-immunoprecipitations in cells. Hip was expressed as a GST fusion protein in *E. coli* and purified using glutathione beads. GST-Hip was able to bind to GRK5, but not GRK2, *in vitro* (Figure 2A). To assess binding of Hip to GRK5 in cells, HA-tagged Hip was expressed at a low level and utilized for immunoprecipitation since a good antibody to immunoprecipitate endogenous Hip was unavailable. Overexpressed

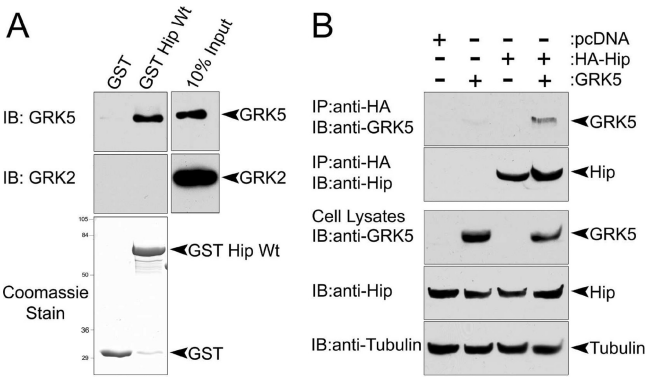

Figure 2. GRK5 binds to Hip. (A) GST and GST-Hip were expressed in *E. coli* and purified using glutathione beads (Coomassie stain). 21 pmol of purified GST or GST-Hip was then incubated with 3 pmol of purified GRK2 or GRK5 for 1.5 h at room temperature with rocking. The beads were washed, and the bound proteins were eluted with the addition of SDS sample buffer. The proteins were then separated by SDS-PAGE, transferred to nitrocellose, and immunoblotted for GRK2 or GRK5. The experiment was performed nine times. (B) HEK293 cells were transiently transfected with pcDNA, HA-tagged Hip Wt, and GRK5 as indicated. Cells were lysed 48 h post-transfection and immunoprecipitated using a polyclonal anti-HA antibody. Immunoprecipitates were then immunoblotted for GRK5 and Hip. Cell lysates were immunoblotted for Hip, GRK4−6, and tubulin expression. The data shown are representative of three independent experiments.

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GRK5 co-immunoprecipitated with HA-tagged Hip (Figure 2B), demonstrating interaction between GRK5 and Hip in cells.

Identification of the GRK5 Binding Site on Hip. Hip can be divided into five domains: an N-terminal domain, a tetracopeptide repeat (TPR) domain, a highly charged region, a region enriched in glycine, methionine, and proline (GGMP), and a C-terminal domain (Figure 3A). The TPR domain and charged region of Hip bind to the ATPase domain of Hsp70, while the GGMP and C-terminal domains bind to the peptide binding domain of Hsp70.[33] Interestingly, previous studies have shown that Hip can bind to the chemokine receptors CXCR2 and CXCR4 in an agonist-dependent manner and that the TPR domain of Hip appears to be involved in the internalization of these receptors.[34] To further define the site where GRK5 binds to Hip, we performed *in vitro* binding assays using various deletion mutants of GST-tagged Hip (Figure 3A). The Hip ΔTPR domain protein did not express well in *E. coli* as a GST-fusion protein and therefore was not evaluated for binding to GRK5. The deletion of the N-terminal domain (Δ1−99) and charged region (Δcharge) of Hip did not result in any significant change in binding to GRK5 (Figure 3B,C). Binding of Hip to the GGMP deletion mutant (ΔGGMP) was reduced compared to wild-type while binding to the C-terminal deletion (Δ303−369) protein was almost completely lost (Figure 3B,C). These data suggest that Hip primarily binds to regions within the C-terminal domain and may form weak interactions with the GGMP domain. To further define where the binding site was located, a series of C-terminal Hip truncation mutants were purified and tested for binding to purified GRK5. C-terminal deletions of 16, 33, or 50 amino acids had a minimal effect on binding while deletion of 66 residues (Δ303−369) effectively disrupted GRK5 binding (Figure 3D,E). This suggests that binding is primarily occurring between residues 303 and 319, which contains the sequence GMPGMAGMPGL-NEILS.

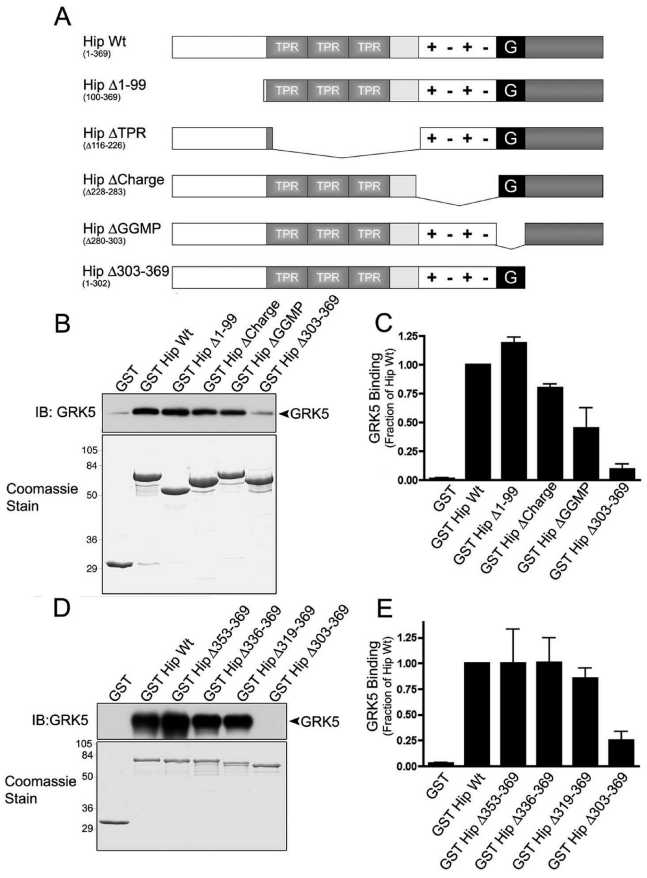

Figure 3. GRK5 binding to Hip. (A) Hip is divided into five domains: the N-terminal domain (residues 1−99, white), three tetracopeptide repeat domains (residues 116−226, gray labeled TPR), a highly charged domain (residues 228−283, white labeled + − + −), a domain enriched in glycine, methionine, and proline (residues 280−303, black labeled G), and a C-terminal domain (residues 303−369, dark gray). (B) GST-tagged Hip domain deletions were expressed in *E. coli* and purified using glutathione beads (Coomassie stain). 21 pmol of GST or GST-Hip deletion mutants were incubated with 3 pmol of purified GRK5 for 1.5 h at room temperature. The beads were then extensively washed, and SDS sample buffer was added to elute the bound proteins. The proteins were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with a monoclonal GRK4−6 antibody. (C) The Western blot in (B) was quantitated using Odysessy software. The data (mean ± SD, n = 3) are from three separate experiments. (D) GST pulldowns were performed as described in (B) using serial truncation mutants of Hip. (E) The Western blot in (D) was quantitated using Odyssey software. The data (mean ± SD, n = 3) are from three separate experiments.

Identification of the GRK5 Phosphorylation Site in Hip. To identify the GRK5 phosphorylation site in Hip, the serial Hip deletion mutants described in Figure 3A were subcloned into a pTrc vector, expressed as hexahistidine-tagged proteins and purified to near homogeneity (Figure 4A). The purified proteins were then phosphorylated by GRK5 *in vitro*. While the Hip Δcharge and ΔGGMP proteins were phosphorylated to a level comparable to wild-type, phosphorylation of the Δ1−99 and ΔTPR proteins was enhanced almost 5-fold compared to wild-type (Figure 4B,C). The enhanced phosphorylation of these two forms of Hip suggests that the optimal conditions for phosphorylation of wild-type Hip by GRK5 are not achieved in the *in vitro* system being used. It seems likely that other factors, such as interacting partners of GRK5 or Hip, may be needed to activate GRK5 and/or promote a conformational change in Hip that is necessary for optimal phosphorylation to occur. These data also suggest that under the correct physiological conditions that there may be multiple sites of phosphorylation by GRK5. Interestingly, the Hip Δ303−369 protein was poorly phosphorylated by GRK5 (Figure 4B,C), suggesting that the primary site of phosphorylation is within this region.

We next attempted to identify the site of phosphorylation by mass spectrometry. Mass spectral analysis of peptides from a trypsin digestion of *in vitro* phosphorylated Hip was unable to detect any phosphorylated peptides, even when phosphopeptide enrichment was performed. However, it was noted that trypsin digestion of Hip resulted in a 7 kDa peptide that spans residues 273−347 (Figure 5A). This fragment significantly overlaps with the C-terminal 303−369 region of Hip that contains the primary site of GRK5 phosphorylation (Figure 4). To determine if phosphorylation of Hip was occurring within the 7 kDa trypsin peptide of Hip, Hip was phosphorylated with radiolabeled ATP. The [^{32}P] phosphorylated Hip was then separated from GRK5 on SDS-PAGE, excised, and digested with trypsin. The peptides that resulted from the trypsinization were then subjected to SDS-PAGE on a 20% polyacrylamide gel. Radioactively labeled bands were detected at 7 kDa as well as in the dye front where most peptides from the Hip digestion

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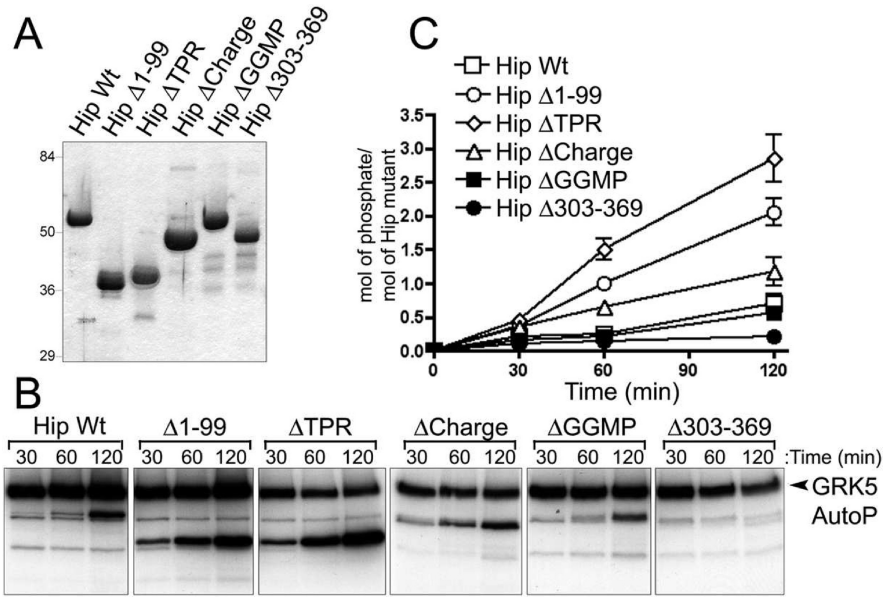

Figure 4. GRK5 phosphorylates Hip between residues 303 and 369. (A) Hexahistidine-tagged Hip deletion mutants were expressed in *E. coli* and purified using Ni^{+} agarose and gel filtration chromatography. 1 μg of purified protein was separated by SDS-PAGE and Coomassie stained. (B) 100 nM of purified GRK5 was incubated with 100 nM of purified Hip deletion mutants for various times at 30 °C in a buffer containing radiolabeled ATP. Samples were separated by SDS-PAGE and subjected to autoradiography. (C) The radioactively labeled bands from (B) were excised and quantitated by scintillation counting to determine the stoichiometry of phosphorylation. Data represent the mean ± SD from five separate experiments.

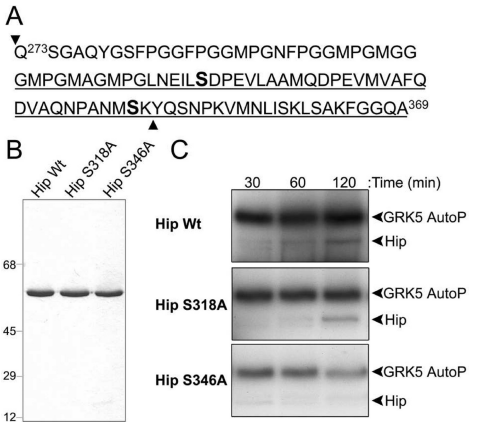

Figure 5. Hip S346A mutant is not phosphorylated by GRK5. (A) The amino acid sequence of Hip between residues 273 and 369 is shown. The underlined portion highlights the residues in the Hip Δ303−369 construct that are deleted. The arrows depict the sites of cleavage by trypsin that result in a 7 kDa fragment. Ser-318 and Ser-346 are in bold. (B) Hexahistidine-tagged Hip wild-type, S318A, and S346A mutants were expressed in *E. coli* and purified using Ni^{+} agarose and gel filtration chromatography. 1 μg of purified protein was separated by SDS-PAGE and Coomassie stained. (C) 100 nM of purified GRK5 was incubated with 100 nM of purified Hip point mutants for various times at 30 °C in a buffer containing radiolabeled ATP. Samples were separated by SDS-PAGE and subjected to autoradiography.

would run (data not shown), suggesting that at least one site of phosphorylation was occurring within residues 273 and 347 and another site was found outside of this region. Taken together with the phosphorylation of the serial deletion mutants of Hip (Figure 4), these data suggest that one of the sites of phosphorylation was within residues 303 and 347. Within this region there are no threonines and two serines, Ser-318 and Ser-346 (Figure 5A). To test whether either of these residues is phosphorylated by GRK5, alanine mutations of the residues were generated and purified (Figure 5B). GRK5 was able to phosphorylate the S318A mutant similar to wild-type Hip but was unable to phosphorylate the S346A mutant (Figure 5C), suggesting that Ser-346 is a primary site of phosphorylation by GRK5.

GRK5 Phosphorylates Ser-346 in Hip in HEK293 Cells. Toward determining the ability of GRK5 to phosphorylate Hip in cells, we attempted to observe phosphorylation of Hip by examining a gel shift in SDS-PAGE. This strategy was unsuccessful at examining Hip phosphorylation *in vitro* or in cells. We were also unsuccessful generating site-specific phosphoantibodies targeting Ser-346. However, the phosphate specific dye, ProQ Diamond, was able to detect the phosphorylation of immunoprecipitated Hip in HEK293 cells (Figure 6A). siRNA knockdown of the endogenous GRKs in HEK293 cells was used to determine which members of this kinase family were able to phosphorylate Hip in cells. Depletion of GRK5 resulted in an ∼35% decrease in Hip phosphorylation, while depletion of GRK3 and GRK6 showed a 20−25% decrease in phosphorylation (Figure 6B,C). These data demonstrate that GRK5 is able to phosphorylate Hip in cells, although it is evident that GRK3, GRK6, and possibly additional kinases also contribute to Hip phosphorylation. To determine if GRK5 phosphorylates Hip at Ser-346, the Hip-S346A mutant was expressed in cells treated with control or GRK5 siRNA. The Hip-S346A mutant consistently exhibited decreased phosphorylation compared to wild-type Hip (Figure 7). Furthermore, knockdown of GRK5 was not able to further decrease the extent of S346A phosphorylation (Figure 7). Taken together, these data suggest that Hip is phosphorylated by GRK5 in cells. As staining of Hip with Pro-Q diamond is unable to distinguish between multiple sites of phosphorylation by different kinases, these data suggest that while Ser-346 is primarily phosphorylated by GRK5, additional sites phosphory-

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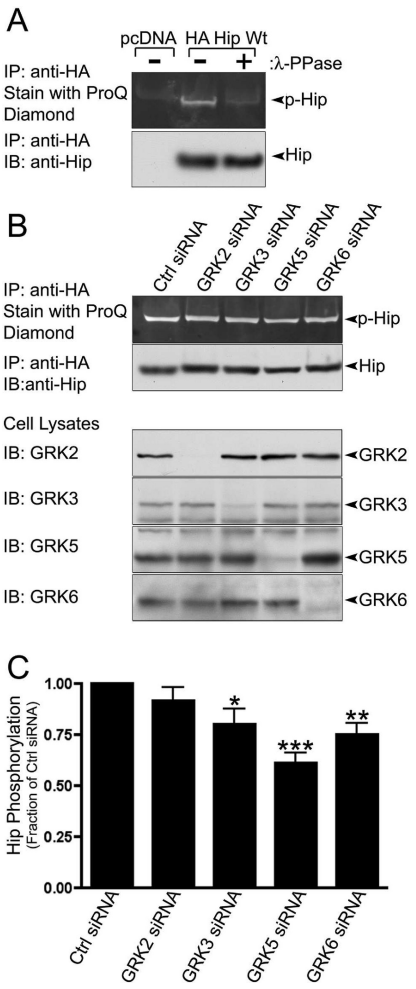

Figure 6. GRK5 phosphorylates Hip in cells. (A) HEK293 cells were transfected with pcDNA3 or HA Hip Wt. 48 h post-transfection, cells were lysed and immunoprecipitated with a monoclonal anti-HA antibody. The immunoprecipitates were then subjected to dephosphorylation by λ-phosphatase for 30 min at 30 °C. The immunoprecipitates were separated by SDS-PAGE, transferred to low-fluorescence PVDF membrane, and stained with Pro-Q Diamond Stain. The stain was visualized using a Typhoon scanner. The PVDF membrane was then immunoblotted for Hip. (B) HEK293 cells were transfected with control (ctrl) or GRK specific siRNA and HA-Hip Wt in pcDNA3. 72 h post-transfection the samples were prepared as described in (A) without λ-phosphatase treatment. Equivalent amounts of cell lysates were probed for GRK expression. (C) The amount of Pro-Q Diamond stain was quantitated using ImageQuant 5.2 software and was normalized to the amount of staining in the control siRNA-treated samples. The data shown are the mean ± SD (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$) from four separate experiments.

lated by other kinases contribute to the global phosphorylation of Hip in cells observed with ProQ Diamond staining.

Phosphorylation of Ser-346 Promotes Internalization of CXCR4. Hip ΔTPR inhibits the internalization of CXCR4 when overexpressed[34] and is phosphorylated by GRK5 better than wild-type Hip *in vitro* (Figure 4). These data suggest that phosphorylation of Hip by GRK5 may modulate the ability of Hip to promote CXCR4 internalization. To examine the role of Hip Ser-346 phosphorylation in modulating CXCR4 internalization, the endogenous expression of Hip was knocked down using a shRNA directed against the 3′ UTR of Hip. This resulted in an ∼75% reduction in endogenous Hip levels (Figure 8A). Hip wild-type, S346A, and S346D mutants along with wild-type FLAG-CXCR4 were then expressed in the shRNA expressing cells (Figure 8A). By rescuing the shRNA knockdown of Hip with exogenous Hip, the effects of the point mutations were investigated without the complication of expression of the endogenous Hip. Upon stimulation of CXCR4 with its ligand, CXCL12, for 30 min in control cells, ∼15% of Flag-tagged CXCR4 was internalized (Figure 8B). The internalization of CXCR4 was almost completely inhibited in the Hip shRNA expressing cells, which was fully rescued by the overexpression of wild-type Hip. Furthermore, expression of the phosphomimetic S346D mutant, but not the S346A mutant, was able to rescue internalization of Hip in the shRNA expressing cells (Figure 8B). These data suggest a novel role of GRK5 in modulating CXCR4 internalization by phosphorylating the nonreceptor substrate Hip at Ser-346.

Toward understanding the role of CXCR4 activation in regulating phosphorylation of Ser-346 in Hip, HEK293 cells expressing CXCR4 and either wild-type or S346A Hip were stimulated with CXCL12, and Hip phosphorylation was assessed using ProQ Diamond staining (Figure 9A,B). Wild-type Hip phosphorylation was decreased ∼25% upon activation of CXCR4, while phosphorylation of the S346A mutant was not affected. Taken together, these data suggest that while the basal phosphorylation of Ser-346 is important for promoting internalization of activated CXCR4, receptor association results in dephosphorylation of Ser-346 and resets the system for another round of GRK5-mediated Hip phosphorylation.

■ DISCUSSION

Traditionally, GRKs phosphorylate activated GPCRs in order to desensitize the receptor and attenuate downstream signaling responses. Recent evidence has suggested that GRKs can phosphorylate non-GPCR substrates, such as tubulin,[14,15] ezrin,[23] arrestin2,[28] and p53,[29] along with several others. The ability of GRKs to phosphorylate nonreceptor substrates suggests that these proteins have many functional roles. Indeed, phosphorylation of ezrin by GRK2 promotes actin rearrangement and influences the internalization of the β_2AR[23] while phosphorylation of arrestin2 by GRK5 inhibits G-protein-independent ERK1/2, signaling downstream of the $5HT_4$ receptor.[28] These studies suggest that GRKs have many undiscovered roles in the signaling and trafficking of GPCRs and possibly in other non-GPCR-related processes.

To attempt to discover novel functions of GRKs, a systematic screen for non-GPCR substrates of GRK5 was initiated. Hip was identified as a novel GRK5 substrate by screening a bovine brain lysate for substrates phosphorylated in a GRK-dependent manner. GRK5 was found to bind to Hip between residues 303 and 319 within the C-terminal portion of the protein and phosphorylate Ser-346 both *in vitro* and in cells. Phosphorylation of Ser-346 appeared to be required for agonist-induced internalization of CXCR4, a GPCR for the chemokine CXCL12. These data suggest a novel function of GRKs in modulating trafficking of a GPCR via phosphorylation of the nonreceptor substrate Hip. Interestingly, GRK5 phosphorylation of Ser-346 does not appear to be the only phosphorylation event occurring on Hip, and further investigation will likely reveal novel kinases and phosphorylation sites that regulate Hip function.

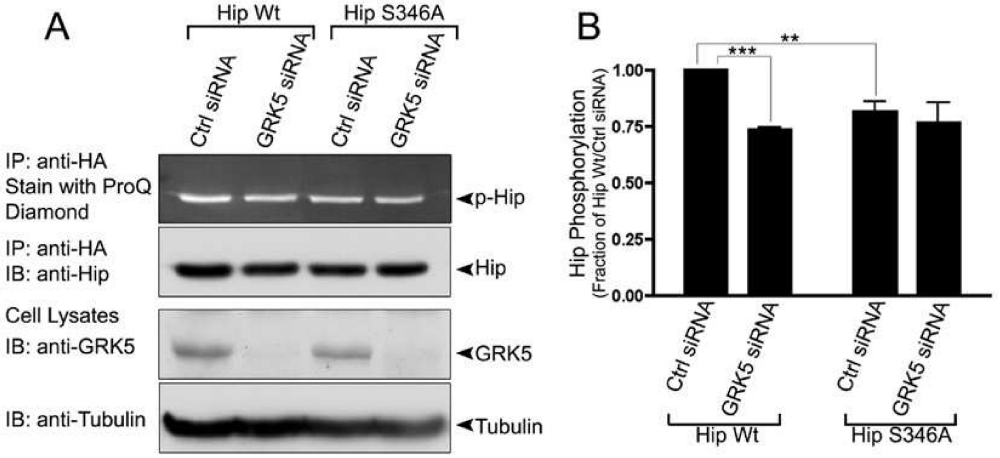

Figure 7. GRK5 phosphorylates Hip at Ser-346. (A) HEK293 cells were transfected with control (ctrl) or GRK specific siRNA and HA-Hip Wt or S346A in pcDNA3. 72 h post-transfection, cells were lysed and immunoprecipitated with a monoclonal anti-HA antibody. The immunoprecipitates were separated by SDS-PAGE, transferred to low-fluorescence PVDF membrane, and stained with Pro-Q Diamond Stain. The stain was visualized using a Typhoon scanner. The PVDF membrane was then immunoblotted for Hip. Equivalent amounts of cell lysates were probed for GRK5 expression. (B) The amount of Pro-Q Diamond stain was quantitated using ImageQuant 5.2 software and was normalized to the amount of staining in the control siRNA treated samples. The data shown are the mean ± SD (**$p \leq 0.01$, ***$p \leq 0.001$) from four separate experiments.

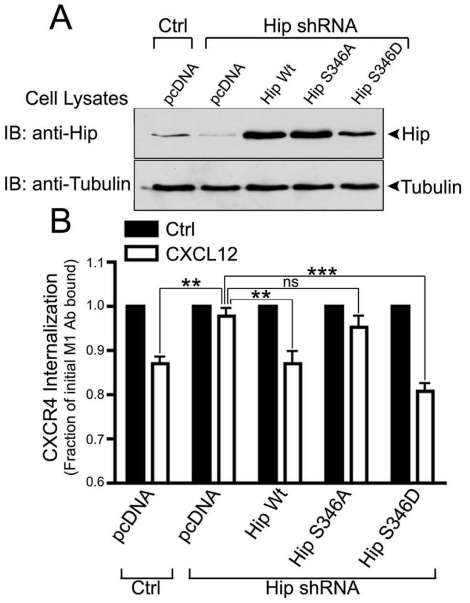

Figure 8. Hip Ser-346 mediates internalization of CXCR4. (A) Control (Ctrl) or Hip shRNA expressing HeLa cells were transiently transfected with FLAG-CXCR4 Wt and pcDNA3-Hip Wt, S346A or S346D. Equivalent amounts of cell lysate were separated by SDS-PAGE, transferred to nitrocellulose, and probed for Hip expression. The membrane was stripped and probed for tubulin as a loading control. (B) Cells transfected as in (A) were serum starved and stimulated with 50 nM CXCL12 for 30 min. The cells were then fixed with 4% PFA, and the remaining amount of surface CXCR4 was determined by cell surface ELISA. The data (mean ± SD, $n = 3$, **$p \leq 0.01$, ***$p \leq 0.001$) are from five separate experiments and are expressed as the fraction of cell surface expression relative to the 0 min untreated control sample.

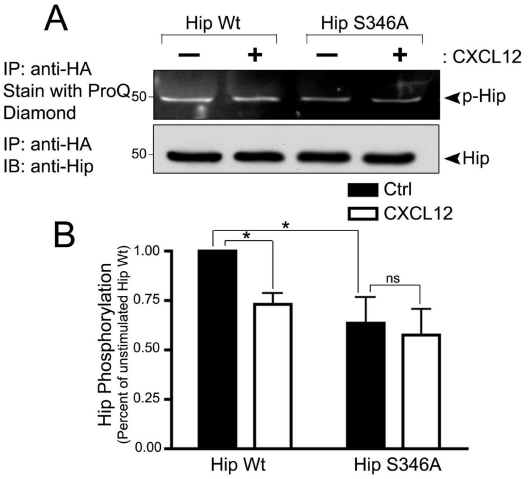

Figure 9. CXCR4 activation promotes dephosphorylation of Hip. (A) HEK293 cells were transfected with Flag-tagged CXCR4 and HA-tagged Wt or S346A mutant Hip. 48 h post-transfection, cells were stimulated with 50 nM CXCL12 for 15 min, lysed, and immunoprecipitated with a monoclonal anti-HA antibody. The immunoprecipitates were separated by SDS-PAGE, transferred to low-fluorescence PVDF membrane, and stained with Pro-Q Diamond Stain. The stain was visualized using a Typhoon scanner. The PVDF membrane was then immunoblotted for Hip. (B) The amount of Pro-Q Diamond stain was quantitated using ImageQuant 5.2 software and was normalized to the amount of staining in the control siRNA-treated samples. The data shown are the mean ± SD (*$p \leq 0.05$) from three separate experiments.

CXCR4 has been implicated in the progression of various disease states, including WHIM syndrome,[35] human immunodeficiency virus-1 entry,[36] and the progression of cancer.[37] Thus, understanding the mechanisms controlling signaling and trafficking of this receptor can lead to novel therapeutics for the treatment of disease. Previous work examining the role of GRKs in signaling and trafficking of CXCR4 has failed to implicate a role for GRK5 in either of these processes. Overexpression of GRK5 in fibroblast cells had no effect on agonist-induced internalization of the receptor,[38] and depletion of GRK5 by siRNA did not affect the ability of CXCR4 to stimulate calcium influx or activate ERK1/2.[30] Although GRK5 has not been implicated in signaling and trafficking of CXCR4 previously, this study suggests a novel role for GRK5 in CXCR4

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trafficking that is independent of receptor phosphorylation and desensitization. There may also be functional redundancy between the GRK family members in modulating Hip phosphorylation. Although GRK5 appears to be the main kinase that phosphorylates Hip, knockdown of GRK3 and GRK6 also had some effect on Hip phosphorylation in cells (Figure 6), and both GRK3 and GRK6 have been implicated in CXCR4 signaling, trafficking, and physiological responses.[30,38,39] Although Hip binds to CXCR4[34] and modulates internalization of the receptor by phosphorylation of Ser-346 (Figure 8), the potential functional redundancy of GRKs in phosphorylating Hip (Figure 6C) makes it unlikely that GRK5 alone regulates CXCR4 internalization through Hip. Taken together, these data suggest that GRKs play an essential role in CXCR4-mediated cellular responses through both canonical and noncanonical mechanisms.

Identification and characterization of novel nonreceptor substrates of GRKs has led to the realization that GRKs play roles in GPCR trafficking other than the canonical phosphorylation and desensitization originally described. Phosphorylation of nonreceptor substrates are likely to play roles in cellular processes that are not related to GPCR signaling, such as protein folding and maturation in the case of Hip phosphorylation by GRK5. Future work identifying nonreceptor substrates of GRKs will likely reveal even more noncanonical roles for this family of kinases.

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ABBREVIATIONS

DMEM, Dulbecco's modified Eagle's Medium; GPCR, G protein-coupled receptor; GRK, GPCR kinase; GST, glutathione *S*-transferase; Hip, heat shock 70 interacting protein; PBS, phosphate buffered saline; PC, phosphatidylcholine; shRNA, short hairpin RNA; siRNA, short interfering RNA; TPR, tetracopeptide repeat.

REFERENCES

(1) Moore, C. A., Milano, S. K., and Benovic, J. L. (2007) Regulation of receptor trafficking by GRKs and arrestins. *Annu. Rev. Physiol. 69*, 451−482.

(2) Marchese, A., Chen, C., Kim, Y. M., and Benovic, J. L. (2003) The ins and outs of G protein-coupled receptor trafficking. *Trends Biochem. Sci. 28*, 369−376.

(3) Drake, M. T., Shenoy, S. K., and Lefkowitz, R. J. (2006) Trafficking of G protein-coupled receptors. *Circ. Res. 99*, 570−582.

(4) Penn, R. B., Pronin, A. N., and Benovic, J. L. (2000) Regulation of G protein-coupled receptor kinases. *Trends Cardiovasc. Med. 10*, 81−89.

(5) Pitcher, J. A., Freedman, N. J., and Lefkowitz, R. J. (1998) G protein-coupled receptor kinases. *Annu. Rev. Biochem. 67*, 653−692.

(6) Carman, C. V., Barak, L. S., Chen, C., Liu-Chen, L. Y., Onorato, J. J., Kennedy, S. P., Caron, M. G., and Benovic, J. L. (2000) Mutational analysis of G$\beta\gamma$ and phospholipid interaction with G protein-coupled receptor kinase 2. *J. Biol. Chem. 275*, 10443−10452.

(7) Onorato, J. J., Gillis, M. E., Liu, Y., Benovic, J. L., and Ruoho, A. E. (1995) The beta-adrenergic receptor kinase (GRK2) is regulated by phospholipids. *J. Biol. Chem. 270*, 21346−21353.

(8) Pitcher, J. A., Fredericks, Z. L., Stone, W. C., Premont, R. T., Stoffel, R. H., Koch, W. J., and Lefkowitz, R. J. (1996) Phosphatidylinositol 4,5-bisphosphate (PIP2)-enhanced G protein-coupled receptor kinase (GRK) activity. Location, structure, and regulation of the PIP2 binding site distinguishes the GRK subfamilies. *J. Biol. Chem. 271*, 24907−24913.

(9) Premont, R. T., Macrae, A. D., Stoffel, R. H., Chung, N., Pitcher, J. A., Ambrose, C., Inglese, J., MacDonald, M. E., and Lefkowitz, R. J. (1996) Characterization of the G protein-coupled receptor kinase GRK4. Identification of four splice variants. *J. Biol. Chem. 271*, 6403−6410.

(10) Stoffel, R. H., Randall, R. R., Premont, R. T., Lefkowitz, R. J., and Inglese, J. (1994) Palmitoylation of G protein-coupled receptor kinase, GRK6. Lipid modification diversity in the GRK family. *J. Biol. Chem. 269*, 27791−27794.

(11) Loudon, R. P., and Benovic, J. L. (1997) Altered activity of palmitoylation-deficient and isoprenylated forms of the G protein-coupled receptor kinase GRK6. *J. Biol. Chem. 272*, 27422−27427.

(12) Kunapuli, P., Gurevich, V. V., and Benovic, J. L. (1994) Phospholipid-stimulated autophosphorylation activates the G protein-coupled receptor kinase GRK5. *J. Biol. Chem. 269*, 10209−10212.

(13) Thiyagarajan, M. M., Stracquatanio, R. P., Pronin, A. N., Evanko, D. S., Benovic, J. L., and Wedegaertner, P. B. (2004) A predicted amphipathic helix mediates plasma membrane localization of GRK5. *J. Biol. Chem. 279*, 17989−17995.

(14) Carman, C. V., Som, T., Kim, C. M., and Benovic, J. L. (1998) Binding and phosphorylation of tubulin by G protein-coupled receptor kinases. *J. Biol. Chem. 273*, 20308−20316.

(15) Pitcher, J.A., Hall, R. A., Daaka, Y., Zhang, J., Ferguson, S. S., Hester, S., Miller, S., Caron, M. G., Lefkowitz, R. J., and Barak, L. S. (1998) The G protein-coupled receptor kinase 2 is a microtubule-associated protein kinase that phosphorylates tubulin. *J. Biol. Chem. 273*, 12316−12324.

(16) Chen, W., Ren, X. R., Nelson, C. D., Barak, L. S., Chen, J. K., Beachy, P. A., de Sauvage, F., and Lefkowitz, R. J. (2004) Activity-dependent internalization of smoothened mediated by beta-arrestin 2 and GRK2. *Science 306*, 2257−2260.

(17) Meloni, A. R., Fralish, G. B., Kelly, P., Salahpour, A., Chen, J. K., Wechsler-Reya, R. J., Lefkowitz, R. J., and Caron, M. G. (2006) Smoothened signal transduction is promoted by G protein-coupled receptor kinase 2. *Mol. Cell. Biol. 26*, 7550−7560.

(18) Freedman, N. J., Kim, L. K., Murray, J. P., Exum, S. T., Brian, L., Wu, J. H., and Peppel, K. (2002) Phosphorylation of the platelet-derived growth factor receptor-beta and epidermal growth factor receptor by G protein-coupled receptor kinase-2. Mechanisms for selectivity of desensitization. *J. Biol. Chem. 277*, 48261−48269.

(19) Pronin, A. N., Morris, A. J., Surguchov, A., and Benovic, J. L. (2000) Synucleins are a novel class of substrates for G protein-coupled receptor kinases. *J. Biol. Chem. 275*, 26515−26522.

(20) Ruiz-Gomez, A., Humrich, J., Murga, C., Quitterer, U., Lohse, M. J., and Mayor, F. Jr. (2000) Phosphorylation of phosducin and phosducin-like protein by G protein-coupled receptor kinase 2. *J. Biol. Chem. 275*, 29724−29730.

(21) Wan, K. F., Sambi, B. S., Frame, M., Tate, R., and Pyne, N. J. (2001) The inhibitory gamma subunit of the type 6 retinal cyclic guanosine monophosphate phosphodiesterase is a novel intermediate

regulating p42/p44 mitogen-activated protein kinase signaling in human embryonic kidney 293 cells. *J. Biol. Chem. 276*, 37802−37808.

(22) Ho, J., Cocolakis, E., Dumas, V. M., Posner, B. I., Laporte, S. A., and Lebrun, J. J. (2005) The G protein-coupled receptor kinase-2 is a TGFbeta-inducible antagonist of TGFbeta signal transduction. *EMBO J. 24*, 3247−3258.

(23) Cant, S. H., and Pitcher, J. A. (2005) G protein-coupled receptor kinase 2-mediated phosphorylation of ezrin is required for G protein-coupled receptor-dependent reorganization of the actin cytoskeleton. *Mol. Biol. Cell 16*, 3088−3099.

(24) Parameswaran, N., Pao, C. S., Leonhard, K. S., Kang, D. S., Kratz, M., Ley, S. C., and Benovic, J. L. (2006) Arrestin-2 and G protein-coupled receptor kinase 5 interact with NFkappaB1 p105 and negatively regulate lipopolysaccharide-stimulated ERK1/2 activation in macrophages. *J. Biol. Chem. 281*, 34159−34170.

(25) Peregrin, S., Jurado-Pueyo, M., Campos, P. M., Sanz-Moreno, V., Ruiz-Gomez, A., Crespo, P., Mayor, F. Jr., and Murga, C. (2006) Phosphorylation of p38 by GRK2 at the docking groove unveils a novel mechanism for inactivating p38MAPK. *Curr. Biol. 16*, 2042−2047.

(26) Ruiz-Gomez, A., Mellstrom, B., Tornero, D., Morato, E., Savignac, M., Holguin, H., Aurrekoetxea, K., Gonzalez, P., Gonzalez-Garcia, C., Cena, V., Mayor, F. Jr., and Naranjo, J. R. (2007) G protein-coupled receptor kinase 2-mediated phosphorylation of downstream regulatory element antagonist modulator regulates membrane trafficking of Kv4.2 potassium channel. *J. Biol. Chem. 282*, 1205−1215.

(27) Martini, J. S., Raake, P., Vinge, L. E., DeGeorge, B. Jr., Chuprun, J. K., Harris, D. M., Gao, E., Eckhart, A. D., Pitcher, J. A., and Koch, W. J. (2008) Uncovering G protein-coupled receptor kinase-5 as a histone deacetylase kinase in the nucleus of cardiomyocytes. *Proc. Natl. Acad. Sci. U. S. A. 105*, 12457−12462.

(28) Barthet, G., Carrat, G., Cassier, E., Barker, B., Gaven, F., Pillot, M., Framery, B., Pellissier, L. P., Augier, J., Kang, D. S., Claeysen, S., Reiter, E., Baneres, J. L., Benovic, J. L., Marin, P., Bockaert, J., and Dumuis, A. (2009) Beta-arrestin1 phosphorylation by GRK5 regulates G protein-independent 5-HT4 receptor signalling. *EMBO J. 28*, 2706−2718.

(29) Chen, X., Zhu, H., Yuan, M., Fu, J., Zhou, Y., and Ma, L. (2010) G-protein-coupled receptor kinase 5 phosphorylates p53 and inhibits DNA damage-induced apoptosis. *J. Biol. Chem. 285*, 12823−12833.

(30) Busillo, J. M., Armando, S., Sengupta, R., Meucci, O., Bouvier, M., and Benovic, J. L. (2010) Site-specific phosphorylation of CXCR4 is dynamically regulated by multiple kinases and results in differential modulation of CXCR4 signaling. *J. Biol. Chem. 285*, 7805−7817.

(31) Kim, C. M., Dion, S. B., Onorato, J. J., and Benovic, J. L. (1993) Expression and characterization of two beta-adrenergic receptor kinase isoforms using the baculovirus expression system. *Receptor 3*, 39−55.

(32) Kunapuli, P., Onorato, J. J., Hosey, M. M., and Benovic, J. L. (1994) Expression, purification, and characterization of the G protein-coupled receptor kinase GRK5. *J. Biol. Chem. 269*, 1099−1105.

(33) Prapapanich, V., Chen, S., Toran, E. J., Rimerman, R. A., and Smith, D. F. (1996) Mutational analysis of the hsp70-interacting protein Hip. *Mol. Cell. Biol. 16*, 6200−6207.

(34) Fan, G. H., Yang, W., Sai, J., and Richmond, A. (2002) Hsc/Hsp70 interacting protein (hip) associates with CXCR2 and regulates the receptor signaling and trafficking. *J. Biol. Chem. 277*, 6590−6597.

(35) Hernandez, P. A., Gorlin, R. J., Lukens, J. N., Taniuchi, S., Bohinjec, J., Francois, F., Klotman, M. E., and Diaz, G. A. (2003) Mutations in the chemokine receptor gene CXCR4 are associated with WHIM syndrome, a combined immunodeficiency disease. *Nature Genet. 34*, 70−74.

(36) Feng, Y., Broder, C. C., Kennedy, P. E., and Berger, E. A. (1996) HIV-1 entry cofactor: functional cDNA cloning of a seven-trans-membrane, G protein-coupled receptor. *Science 272*, 872−877.

(37) Zlotnik, A. (2006) Involvement of chemokine receptors in organ-specific metastasis. *Contrib. Microbiol. 13*, 191−199.

(38) Balabanian, K., Levoye, A., Klemm, L., Lagane, B., Hermine, O., Harriague, J., Baleux, F., Arenzana-Seisdedos, F., and Bachelerie, F. (2008) Leukocyte analysis from WHIM syndrome patients reveals a pivotal role for GRK3 in CXCR4 signaling. *J. Clin. Invest. 118*, 1074−1084.

(39) Fong, A. M., Premont, R. T., Richardson, R. M., Yu, Y. R., Lefkowitz, R. J., and Patel, D. D. (2002) Defective lymphocyte chemotaxis in beta-arrestin2- and GRK6-deficient mice. *Proc. Natl. Acad. Sci. U. S. A. 99*, 7478−7483.